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14. ABSTRACT

Protocadherin-PC (PCDH-PC) is a male-specific gene that is highly upregulated in androgensensitive prostate cancer cells by androgen deprivation. Based on our preliminary work, we proposed that PCDH-PC protects prostate cancer cells from cell death subsequent to hormone therapy. Work in the project seeks to develop shRNA and antisense oligonucleotide agents that block PCDH-PC expression and to test whether suppression of PCDH-PC expression increases prostate cancer cell death under androgen-free conditions and whether this action blocks the development of hormone-resistant prostate cancers in mice xenografted with human prostate cancer cells. During the previous period we successfully developed shRNA and antisense oligonucleotide agents that target and suppress PCDH-PC expression. These agents are surprisingly toxic for cultured androgen-sensitive prostate cancer cells even when androgens are present and the results suggest that low level expression of PCDH-PC might contribute to prostate cancer cell survival.

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Prostate Cancer; Hormone-Therapy; Hormone-Refractory Prostate Cancer; shRNA; Antisense Oligonucleotides; Apoptosis

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Annual Progress Report (I + II)

DOD Project: DAAMRDC W81XWH-06-00

Title: *Targeted Elimination of PCDH-PC Expression for Control of Hormone-Resistant Prostate Cancer*

Principal Investigator: Ralph Buttyan, Ph.D.

Progress Period: November 2005 to November 2007

Introduction: Protocadherin-PC (PCDH-PC) is an androgen-repressed gene encoded on the human Y-chromosome that is over-expressed in prostate cancer cells that were selected for resistance to apoptosis (1). Transfection of this gene product back into prostate cancer cells induces apoptosis- and hormone-resistance (1,2). PCDH-PC is also highly upregulated in androgen-sensitive human prostate cancer cells when they are exposed to androgen-free conditions (1). In our preliminary studies, we found that siRNAs that target and suppress the expression of PCDH-PC were able to dramatically increase the number of prostate cancer cells killed by exposure to androgen-free conditions. Based upon these results, we proposed that agents that target and block PCDH-PC expression might be used in conjunction with hormonal therapy to increase response and survival of advanced (metastatic) prostate cancer patients. The work in this project will develop strategies to suppress PCDH-PC expression in prostate cancer cells based on shRNA and antisense oligonucleotide targeting. We will then test whether cultured human prostate cancer cells (LNCaP) that are crippled with regards to their ability to upregulate PCDH-PC (by transfection with a PCDH-PC specific targeting shRNA), are killed when they transferred to androgen-deprived medium and whether these cells are unable to form hormone-refractory tumors when xenografted into male immunodeficient mice that are subsequently castrated. We will also test whether the treatment of LNCaP-xenografted mice with antisense oligonucleotides that target PCDH-PC, prevent these mice from developing hormone refractory tumors after they are castrated. In summary, the work in this project will pre-clinically test the idea that PCDH-PC targeting strategies might be a useful adjuvant therapeutic when advanced prostate cancer patients undergo hormonal therapy.

Body: This project has 4 Specific Aims and progress will be discussed for each Aim.

Specific Aim 1**.** Design shRNA expression plasmids that suppress PCDH-PC expression in LNCaP cells and isolate transfected variants of the LNCaP cell line that are unable to induce PCDH-PC expression when cultured in androgen-free medium.

Work Done: We first designed 3 different PCDH-PC shRNA targeting vectors in the pGsh1-GFP lentiviral vector that incorporate the 19 basepair targeting sequences of our already developed PCDH-PC targeting siRNAs (3) into a hairpin expression structure. The short hairpin inserts and their orientation were confirmed by vector sequencing. These vectors were then transfected into LNCaP cells and the Flow-Activated Cell Sorter was used to capture GFP-expressing cells subsequent to transfection. The transfected cells were expanded and frozen, along with LNCaP cells expressing a control (no shRNA insert) vector. The three transfected shRNA cell variants were then tested for reduction of PCDH-PC expression when grown in androgen-free medium (phenol red-free RPMI-1640 supplemented with 10% charcoal-stripped fetal bovine serum (CS-FBS). After 7 days growth in this medium, RNAs were collected from these cells and they were compared to RNAs extracted from the control transfected LNCaP cells for expression of the PCDH-PC mRNA by the use of a semi-quantitative RT-PCR technique. There was no significant difference in levels of PCDH-PC mRNA

expressed between the cells with putative targeting shRNA compared to cells with control (empty) vector (less than 10% difference in PCDH-PC mRNA levels). We concluded from this work that the sequences that were suitable for siRNA targeting of PCDH-PC are not suitable for shRNA-based targeting.

We then took advantage of the services of Origene, Inc., and obtained 4 different shRNA vectors (#310597-310600) that hypothetically target PCDH-PC (based upon their computer analytical programs). These vectors utilize a 29 base targeting sequence rather than the 19 base targeting sequence that was present in our own shRNA vectors and the company claims that the efficiency of gene suppression is significantly enhanced by the longer targeting sequences. The company also provided two vectors (#20003 and 30003) that contain non-targeting shRNA hairpins to serve as controls. All of the shRNAs are in puromycin-selectable retroviral vectors and they can be used to produce defective retroviral particles for more efficient delivery to cells. In Figure 2, below, we show the results of a Western blot procedure from an experiment in which a myc-tagged PCDH-PC expression vector was transfected into 293T cells (does not express PCDH-PC) along with equal aliquots of the control vectors (#30003 or 20003) or any of 4 PCDH-PC targeting shRNA vectors (#310597-600) . Forty-eight hrs after transfection, cell extracts were prepared and aliquots containing equal amounts of protein were co-electrophoresed on an SDS-PAGE gel. The Western blot made from this gel was probed with antibody that recognizes the myc-tag. When compared to the extracts from the controls co-transfected with non-targeting shRNA vectors, our results indicate that the PCDH-PC shRNA targeting vectors 310598 and 310600 are the most effective in suppressing expression of the PCDH-PC protein. These results were reproduced when the test cell system was the LNCaP (human prostate cancer) cell line (not shown).

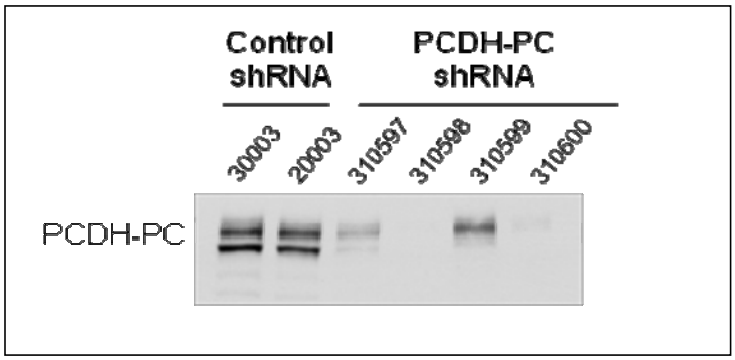


Figure 1 (Above). Reduction of PCDH-PC expression by shRNA targeting vectors. Western blot shows suppression of PCDH-PC expression in 293T cells by PCDH-PC targeting shRNA vectors (#310597-310600) when co-transfected along with a myc-tagged cDNA expression vector containing PCDH-PC cDNA. Cell extracts were prepared 48 hrs after co-transfection and were electrophoresed on an SDS-PAGE gel and blotted onto a filter. The filter was probed with an anti-myc antibody. The levels of myc-tagged PCDH-PC protein in cells co-transfected with control shRNA vectors (#30003 and 2003) is sharply contrasted with the effects of co-transfection with any of the PCDH-PC targeting vectors (#310597-310600). However the effects of two of the shRNA targeting vectors (#310598 and 310600) were especially pronounced with virtual complete surpression of PCDH-PC expression in these cells. These results were also reproduced in LNCaP cells.

Based upon our results, we then proceeded in an attempt to produce stable transfected variants of LNCaP cells using each of these vectors. Each of the vectors described above (control and PCDH-PC targeting) were individually transfected into LNCaP cells using a lipofectamine procedure. Following transfection, cells were selected in a puromycin containing medium to obtain clonal transformants. Remarkably, the cells transfected with either of the control shRNA vectors produced numerous puromycin-resistant colonies (> 200/dish) whereas only the cells transfected with PCDH-targeting #310599 or #310597 vectors produced any stable surviving colonies (<50 and 2, respectively). Cells transfected with shRNA-targeting vectors #310598 and #310600 did not produce any surviving colonies. This experiment was repeated with similar results. These experiments are remarkable since the most effective PCDH-PC-targeting shRNAs (#310598 and #310600) do not support puromycin-resistant colony following transfection into LNCaP cells and this may be because they are suppressing the low level expression of this gene product in these cells.

Work During the Next Period: We are transfecting the 2 most effective PCDH-PC shRNA targeting vectors into PC-3 cells which do not express PCDH-PC since they lack the Y chromosome. If they we are able to efficiently obtain surviving clones with these cells, this will support the idea that the shRNA targeting PCDH-PC is lethal even for cells that express very low levels of this gene. Our results with these shRNA vectors in LNCaP cells also suggests the need for a regulatable shRNA expression vector to complete the work proposed in Aim 1. To address this, during the next year, we will construct tetracycline-regulatable shRNA targeting vectors using the hairpin sequences in our most effective shRNA vectors and we believe that these vectors will enable us to complete the work proposed in Aim 1 as described.

Specific Aim 2. Design and test antisense oligonucleotides (ASOs) that suppress PCDH-PC expression in prostate cancer cells.

Work Done: We used the sequence of our most successful siRNA (#181, ref 2) to design a PCDH-PC-targeting antisense oligonucleotide (19 bases). Figure 2 (below), shows the results obtained when this ASO was transfected into LNCaP cells that were previously transfected with a myc-tagged PCDH-PC expression plasmid. Our results show that this ASO is effective in silencing PCDH-PC expression at 200 μM concentrations or greater. We believe that this data indicates that ASO #181 suitably targets PCDH-PC at concentrations above 200 μM and completes the work described for Specific Aim 2.

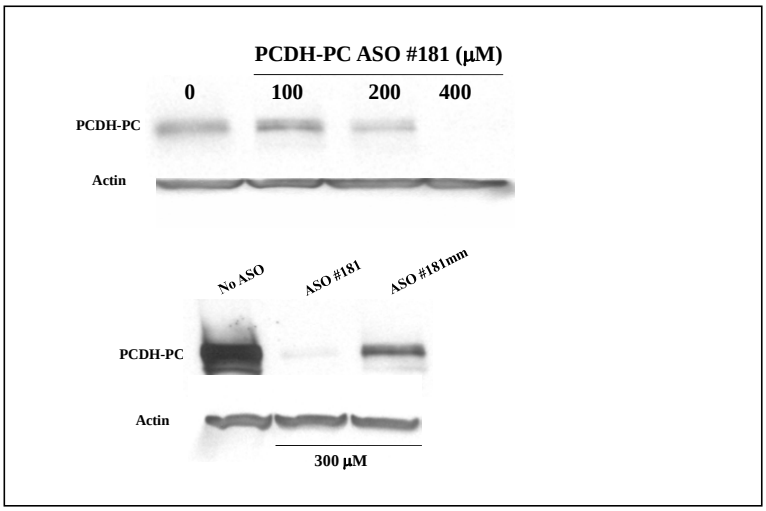


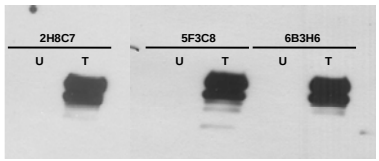
Figure 2 (Above). Reduction of PCDH-PC expression by ASO #181. (Top Panel). ASO #181 was transfected into LNCaP cells 24 hrs subsequent to transfection with a myc-tagged PCDH-PC expression vector. 48 hrs later extracts were made of the cells and the extracts were analyzed by Western blot for expression of myc-tagged PCDH-PC protein (110 kd). Results show significant reduction of PCDH-PC expression at 200 uM and higher concentration. (Bottom Panel) The experiment was repeated at a 300 uM ASO concentration and compared to the same concentration of the 181-mm ASO (containing 4 mismatched bases). Results show significant specificity to ASO 181-mediated reduction of PCDH-PC expression.

Specific Aim 3. Test PCDH-PC expression reduction strategies (shRNA or ASO targeting) for ability to induce death of LNCaP cells *in vitro* in androgen-free medium.

Work Done: In our work in Specific Aim 1, our effective PCDH-PC shRNA targeting vectors appear to be lethal for prostate cancer cells even when grown in medium (standard RPMI + 10% FBS) that contains very low levels of androgen. Our results indicate the possibility that even the low levels of PCDH-PC expressed in LNCaP cells is important for their survival. Therefore, we are now attempting to create a tetracycline-inducible shRNA targeting vector that will allow us to better control the expression of the PCDH-PC targeting shRNAs in LNCaP cells and adjust the PCDH-PC suppression so that we can complete this experiment as described. For these purposes, however, we have also made progress in developing our own monoclonal antibodies that detect (untagged) PCDH-PC protein and these antibodies will be used in completing this Specific Aim as described. We had not proposed this work in our original project since our collaborators in France (the Laboratory of Dr. Dominique Chopin of INSERM-038, Creteil) had already developed a monoclonal antibody that was able to detect untagged PCDH-PC protein on Western blots and in tumor cells by immunohistochemistry. However, our progress in this Aim was complicated due to the fact that Dr. Chopin passed away in 2006 and his laboratory was disbanded. After this, we were no longer able to obtain this critical research resource. In order to overcome this problem, we contracted with a company, Promab, Inc in Albany California, to produce our own monoclonal antibodies against peptides from PCDH-PC. The company provided us with supernatants from 20 different clones derived after injection of two different peptides from the C-terminal region of PCDH-PC. Each of

these supernatants were tested in a Western blot assay containing extracts of 293T cells that were transiently transfected with a myc-tagged PCDH-PC cDNA or with empty vector. Our assays identified 3 different clones whose supernatants easily recognize the appropriate molecular weight bands on Western blots (Figure 3 below) and these supernatants were unreactive with control (untransfected) cell extracts (not shown). One of the antibodies (5F3C8) was also successful in detecting PCDH-PC protein in untransfected LNCaP cells as well as in LNCaP cells grown in androgen-free medium (not shown). This antibody will be used to monitor expression of PCDH-PC in all of our subsequent experimental work.

Figure 3. Western blot showing reactivity of supernatants from 3 mouse clones that recognize the 110 kd PCDH-PC protein expressed in transfected 293T cells.



U = Untransfected 293T Cell Extract
T = 293T Cells Transfected with PCDH-PC

We will be receiving these monoclonal cells for our own repository and will contract with the company for production of an antibody enriched fraction from each of these three clones for use in our further experimentation. We believe that this is a major break-through in that we will now have unrestricted access to antibodies that can directly recognize the PCDH-PC protein (on Western blots) so that we can utilize these antibodies in experiments involving human prostate cancer cell lines that have not been transfected with tagged versions of the cDNA. We are also presently assessing their status as immunohistochemical detectors of PCDH-PC protein expression in prostate cancer cell lines. If this proves useful, we can use them to assess PCDH-PC status in human prostate cancer tissue microarrays. The latter work will allow us to determine whether the expression of this protein in actual human prostate tumor cells correlates with significant clinical parameters of the patients from whom the specimens were derived (tumor stage, grade or patient response to therapy).

Work During the Next Period: We are constructing a tetracycline-inducible PCDH-PC targeting shRNA vector that will enable us to modulate the suppression of PCDH-PC expression in prostate cancer cells and complete the Aim as described.

Specific Aim 4 Proof-of-principle and pre-clinical testing of targeted PCDH-PC expression knockout combined with androgen deprivation as a strategy for prostate cancer therapy using mouse xenograft tumor models.

No work was done on this Specific Aim during the last period. This work will be carried out during the next period of the project.

Key Research Accomplishments

- Testing and identification of effective shRNA targeting vectors that suppress expression of PCDH-PC in prostate cancer cells
- Design and testing of an effective anti-sense oligonucleotide that suppresses expression of PCDH-PC in prostate cancer cells

- Demonstration that shRNAs targeting PCDH-PC expression in prostate cancer cells effectively kill these cells even when androgens are present
- Development of highly sensitive monoclonal antibodies that allow detection of native PCDH-PC protein on Western blots

Reportable Outcomes

Published Manuscripts:

1. Yang, X., Chen, M.-W., Terry, S., Vacherot, F., Bemis, D.L., Capodice, J., Guo, Y. and Buttyan, R. (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. *Oncogene*, 25: 3436-3444.
2. Terry, S., Queires, L., Gil-Diez-de-Medina, S., Chen, M.-W., de la Taille, A., Allory, Y., Tran, P.-L., Abbou, C.C., Buttyan, R. and Vacherot, F. (2006) Protocadherin-PC promotes androgen-independent prostate cancer cell growth. *Prostate*, 66: 1100-1113.

Biological Resources:

1. shRNA expression vectors (2) that suppress PCDH-PC expression in prostate cancer cells.
2. Antisense Oligonucleotide (#181) that suppresses PCDH-PC expression in prostate cancer cells.
3. Monoclonal antibodies (3) that detect PCDH-PC protein on Western blots.

Conclusions

Our hypothesis is that reduction/suppression of PCDH-PC expression in prostate cancer cells will enhance cancer cell killing when combined with hormone therapy. Work in this project has so far led to the development of two types of biological agents that specifically target and suppress PCDH-PC expression in prostate cancer cells (shRNA and antisense oligonucleotide) and these agents are, at least, useful tools for identifying the role of PCDH-PC in the development of hormone-refractory prostate cancer. Testing during the next period of this project will help us understand whether these agents might have potential as adjuvant therapeutics that might be used, in conjunction with hormone therapy to improve treatment of advanced prostate cancer. Other work in this project has led to the development of new monoclonal antibodies that recognize PCDH-PC protein and these antibodies might be useful to characterize PCDH-PC expression in human prostate tumor tissues and to correlate this gene marker with disease characteristics.

References

1. Chen, M.-W., Vacherot, F., de la Taille, A., Shen, R., Gil-Diez-de-Medina, S., Chopin, D.K., Friedman, R. and Buttyan, R. (2002) Emergence of protocadherin T6 expression during the acquisition of apoptosis resistance by human prostate cancer cells. *Oncogene*, 21: 7861-7871.
2. Terry, S., Queires, L., Gil-Diez-de-Medina, S., Chen, M.-W., de la Taille, A., Allory, Y., Tran, P.-L., Abbou, C.C., Buttyan, R. and Vacherot, F. (2006) Protocadherin-PC promotes androgen-independent prostate cancer cell growth. *Prostate*, 66: 1100-1113.
3. Yang, X., Chen, M.-W., Terry, S., Vacherot, F., Chopin, D., Bemis, D., Kitajewski, J., Benson, M.C., Guo, Y. and Buttyan, R. (2005) A human- and male-specific protocadherin that acts through the wnt signaling pathway to induce neuroendocrine transdifferentiation of prostate cancer cells. *Cancer Res*. 65: 5263-5271.